Plant Physiol. (1982) 70, 283–286
0032-0889/82/70/0283/04/$00.50/0

Metabolism of Exogenous Indoleacetic Acid to Its Amide Conjugates in *Cucumis sativus* L. [1,2]

Received for publication October 5, 1981 and in revised form March 1, 1982

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ABSTRACT

Incubation of hypocotyl segments of light-grown *Cucumis sativus* L. in 0.1 millimolar 3-indoleacetic acid for 16 hours led to the formation of indoleacetylaspartate and indoleacetylglutamate. There was no evidence for the formation of other conjugates of 3-indoleacetic acid with individual amino acids during the period from 4 to 48 hours of incubation. Indoleacetylglutamate reached its maximum concentration after about 4 hours of incubation and indoleacetylaspartate after about 8 hours. These levels remained unchanged for at least 40 hours. Indoleacetylaspartate caused small increases in cucumber hypocotyl segment growth at high concentrations, 1 millimolar being more effective than 0.1 millimolar.

The roles of amide bound auxins such as IAAsp[3] have not been determined with certainty. The formation of IAAsp from exogenous IAA is well documented, beginning with the initial report by Andreae and Good (1) for pea seedlings. The formation of IAGlu was first reported for excised tomato roots (11) and later, more convincingly, for crown gall callus from *Parthenocissus tricuspidata* (3). IAAsp and IAGlu were not the major amide bound auxins produced in *Parthenocissus* crown gall tissue, for indoleacetylvaline, indoleacetylalanine, and indoleacetylglycine were produced in substantially greater amounts (3). Amide conjugates of 2,4-D and of naphthaleneacetic acid have also been studied; and it has been suggested that for both of these synthetic auxins, the glutamate conjugate is formed first, with the aspartate conjugate being formed from the glutamate conjugate rather than directly from 2,4-D or naphthaleneacetic acid (5, 6).

In preparation for a study of the native bound auxins of the cucumber shoot, we have examined the production of amide bound auxins following the application of isotopically labeled IAA to isolated stem segments. Our intention was to learn which amide bound auxins the plant can make before undertaking the more difficult task of studying which ones are formed when the plant is not challenged by exogenous auxin. We observed the appearance of only two conjugates of IAA with individual amino acids, namely IAAsp and IAGlu. Any other conjugates of IAA with common amino acids that may have been formed were produced in quantities more than an order of magnitude lower than those of IAGlu, the less abundant of the two conjugates observed, at least during the first 4 d following application of IAA. Several other labeled compounds were formed during that period, but none of them were further characterized.

MATERIALS AND METHODS

Plant Material and Growth Tests. Seeds of *Cucumis sativus* L. (cv. Straight Eight, Burpee Seed Co.) were soaked for 1 h in tap water, sown in moist vermiculite, and allowed to develop for 4 d in a growth chamber at 27°C on a daily photoperiodic regime of 16 h light and 8 h darkness. Light was from mixed incandescent and cool-white fluorescent sources. Stem segment growth tests were performed as described (10). Segments initially 2.0 cm in length, taken immediately below the cotyledons, were incubated for 20 h in the dark in 2 ml of medium in Stender dishes. Final lengths were measured to the nearest 0.5 mm.

Amide Bound Auxins. A sample of IAAsp, initially prepared by Calbiochem and kindly provided by Dr. Jack Valdovinos, was used for the growth test. Samples of radioactively labeled IAAsp and IAGlu were prepared by the methods of Hollenberg *et al.* (8), modified after Mollan *et al.* (9). The *p*-nitrophenyl ester of IAA (9) was added to a solution of tetramethylguanidine and the appropriate ^{3}H- or ^{14}C-labeled amino acid in aqueous methanol.

Treatment with Radiolabeled IAA. Stem segments 2 cm in length were cut from the region immediately below the cotyledons. The segments were placed in groups in shell vials. Droplets of labeling solution, containing either [^{3}H(G)]IAA (New England Nuclear) or [2′-^{14}C]IAA (Research Products International), were placed on the cut ends of the segments and allowed to sink into the tissue, whereupon the vials were filled with enough unlabeled 0.1 mM IAA to cover the tissue. The segments were then vacuum infiltrated with this unlabeled solution, transferred with the IAA solution into a Petri dish containing a circle of filter paper (the segments were not submerged), and incubated in the light at room temperature.

Extraction, Separation, and Analysis of Labeled Compounds. Following incubation, the stem segments were ground in a glass homogenizer with successive 2-ml aliquots of methanol until the residue was colorless. The homogenate was filtered through filter paper, and the filtrate was evaporated under vacuum in a rotary evaporator until about 0.5 ml of an aqueous residue remained. After the addition of 100 μl of methanol, the residue was filtered through a Whatman 'Sep-Pak' (a cartridge containing a small amount of a reverse phase C_{18} stationary phase), as were two rinsings of the evaporator flask (each about 0.5 ml of 10% [v/v] aqueous methanol). The Sep-Pak removed pigments as well as fine particulates which might have clogged the HPLC column. The combined filtrates from the Sep-Pak constituted the sample for subsequent fractionation and analysis.

Aliquots of the filtrate were subjected to HPLC in a Varian Model 5000 as described by Hollenberg *et al.* (8). We used a Varian MCH-10, 30 cm × 4 mm, reverse phase C_{18} column. The solvents were 1% glacial acetic acid (A) and acetonitrile (B). These were supplied according to the following program: pure A from 0

[1] Dedicated to the memory of William Sermolino Hillman: friend, scientist, and poet.

[2] Supported by National Science Foundation Grant PCM-7726476 (to W. K. P.).

[3] Abbreviations: IAAsp, indoleacetylaspartate; IAGlu, indoleacetylglutamate.

to 5 min, a linear gradient from 0 to 30% B from 5 to 30 min, and a linear gradient to 100% B from 30 to 45 min. The flow rate was 1 ml/min. Prior to injection into the HPLC, the samples were mixed with [^{3}H] or [^{14}C]IAA, IAAsp, and IAGlu in concentrations roughly commensurate with the concentrations of those compounds present in the extract. Fractions (20 s) were collected from the HPLC, and each was added to 10 ml of Bray's solution for counting in a Beckman Series 9000 liquid scintillation counter with a data-reduction module which calculated dpm.

Base Hydrolysis. An aliquot (50 μl) of a plant sample was combined with labeled markers (IAA, IAAsp, and IAGlu, 5 μl each), 5 μl of 10 N NaOH was added, and the reaction mixture was stirred for 1.5 h at room temperature. The mixture was then neutralized with approximately 5 μl of glacial acetic acid, and a 70-μl sample was injected into the HPLC.

Methyl Esterification. A 50-μl sample was combined as above with ^{14}C-labeled markers. Freshly prepared diazomethane in diethyl ether was added until a yellow color persisted in the reaction mixture, whereupon the reaction was quenched with a small amount of glacial acetic acid. A 70-μl sample was injected into the HPLC.

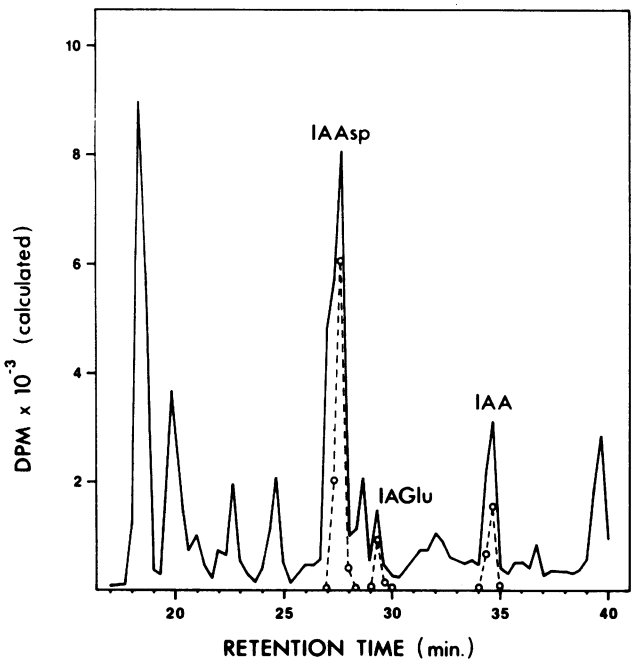

FIG. 1. HPLC of radioactive metabolites in extracts of cucumber hypocotyls incubated with [^{3}H(G)]IAA. Dotted lines show positions of authentic [^{14}C]IAA (34.7 min), IAA-[^{14}C]Asp (27.7 min), and IAA-[^{14}C]Glu (29.3 min).

RESULTS

Metabolites of Exogenous [^{3}H]IAA. Segments of 25 stems were vacuum infiltrated with a labeling solution containing 2.3 μCi of [^{3}H(G)]IAA and unlabeled IAA at a concentration of 0.1 mM. After 16 h of incubation, the treated segments were homogenized as described above. Once the extract had been partially purified, 100 μl of extract were combined with 5 μl each of solutions containing [^{14}C]IAA, IAA-[^{14}C]Asp, and IAA-[^{14}C]Glu. A 70-μl aliquot of the mixture was injected into the HPLC. Fractions (20 s) of the eluate were assayed for radioactivity in the liquid scintillation counter, with the results shown in Figure 1. More than 10 significant peaks of ^{3}H-radioactivity were observed in the fractions collected between 15 and 40 min. This was the range previously determined to be the one in which the amide conjugates of IAA eluted (8). The peak which cochromatographed with IAA represented a small minority of the radioactive material present, indicating that metabolism of IAA was rapid during the period. A small peak of tritiated material cochromatographed with authentic IAGlu, and one of the two largest peaks cochromatographed with IAAsp.

Effects of Base Hydrolysis. Another aliquot of the extract was treated with base as described in "Materials and Methods." The conditions employed were such as to hydrolyze ester bound auxins but not amide bound auxins (2). When the hydrolyzed sample was subjected to HPLC along with [^{14}C]IAA, IAAsp, and IAGlu, all but six of the peaks of ^{3}H-radioactivity seen in Figure 1 had shifted or disappeared—most of the metabolites seen in Figure 1 were base labile. Three of the unchanged peaks corresponded to

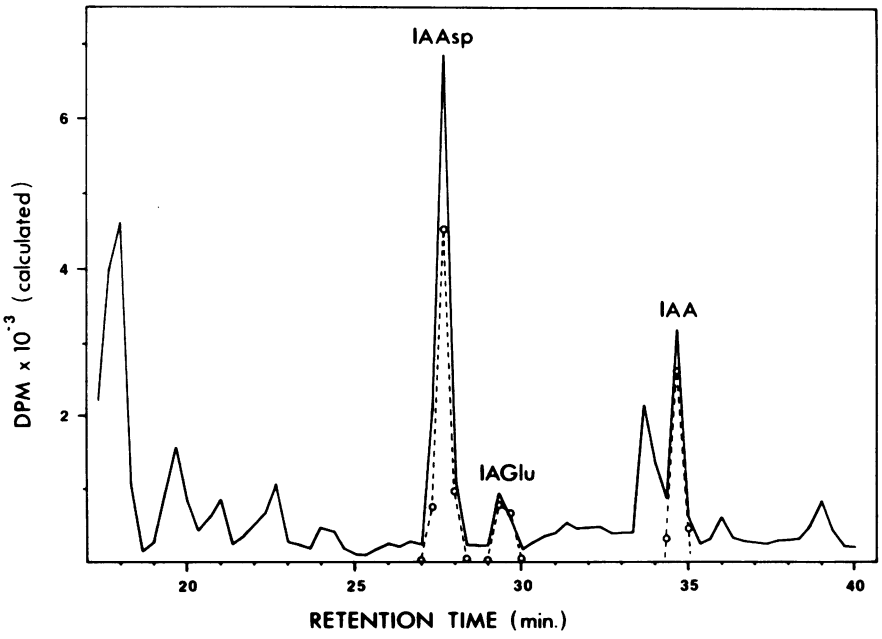

FIG. 2. HPLC of radioactive metabolites of [^{3}H(G)]IAA, after treatment of extract with 1 N NaOH. Dotted lines show positions of authentic [^{14}C]IAA (34.7 min), IAA-[^{14}C]Asp (27.7 min), and IAA-[^{14}C]Glu (29.3 min).

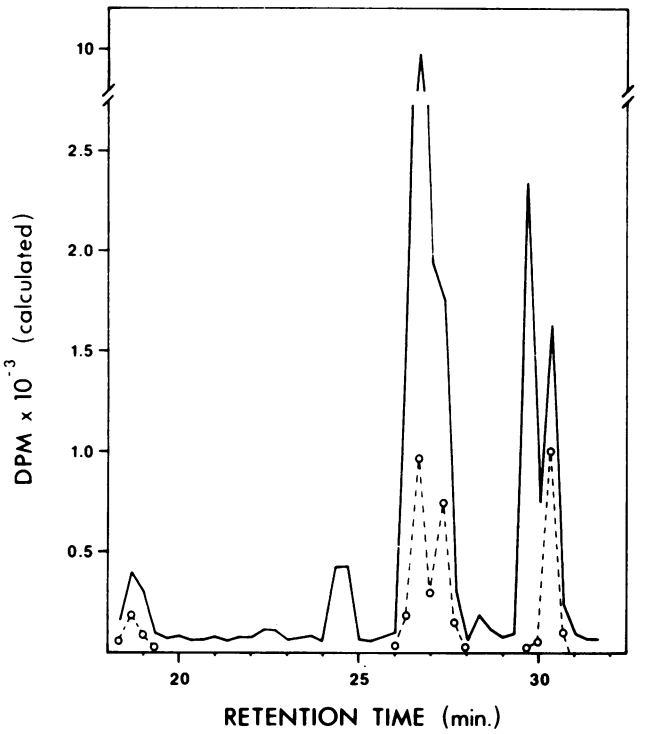

FIG. 3. HPLC of radioactive metabolites of [^{3}H(G)]IAA, after treatment of extract with diazomethane. Dotted lines show positions of authentic methyl ester of [^{14}C]IAA (30.3 min) and presumed methyl esters of IAA-[^{14}C]Asp and IAA-[^{14}C]Glu (both between 26 and 28 min).

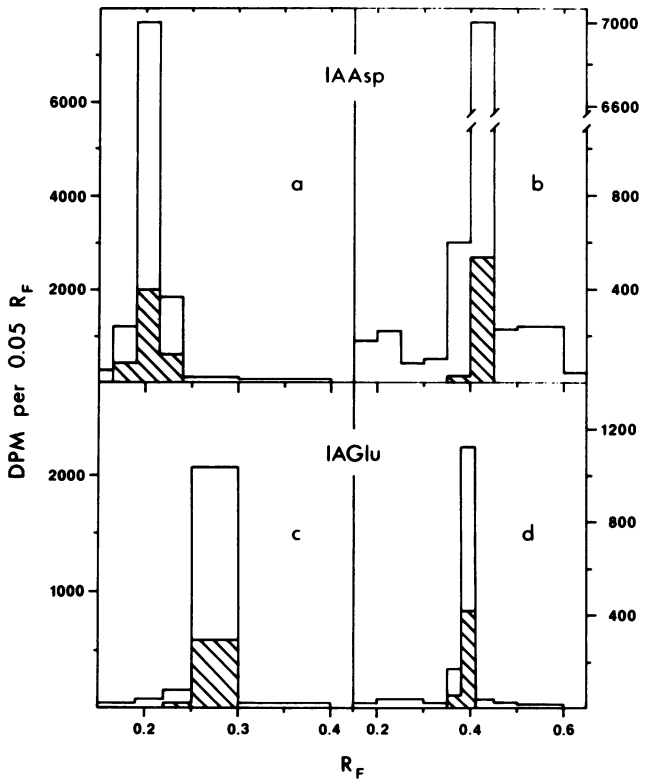

FIG. 4. TLC of presumed [^{3}H]IAAsp and [^{3}H]IAGlu peaks obtained from HPLC as in Figure 1. Hatched bars show positions of authentic IAA-[^{14}C]Asp (top) and IAA-[^{14}C]Glu (bottom). Solvents are isopropanol:ammonia:water (a and c) and chloroform:ethyl acetate:formic acid (b and d).

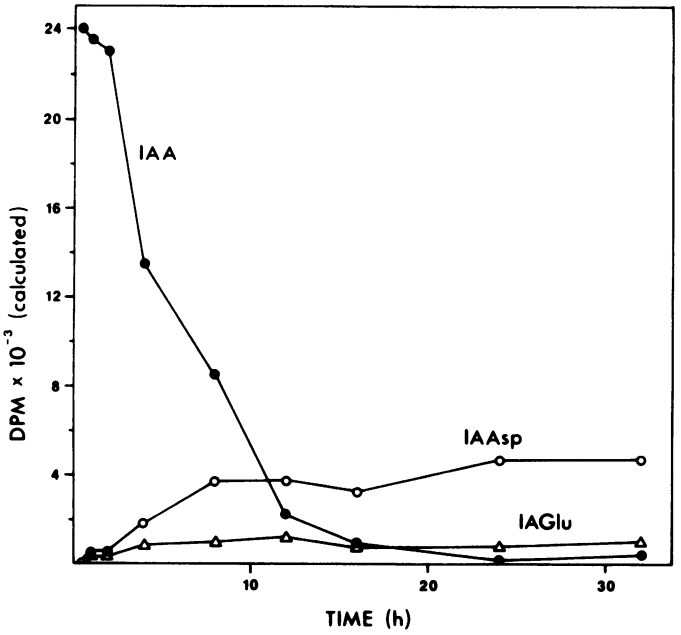

FIG. 5. Levels of [^{14}C]IAA (●), [^{14}C]IAAsp (○), and [^{14}C]IAGlu (△) during incubation of cucumber hypocotyl segments with [2′-^{14}C]IAA. Values represent peak heights replotted from graphs as in Figure 1.

Table I. *Effect of IAAsp on Elongation of Cucumber Hypocotyl Segments*

	[IAAsp][a]	Elongation[b]
	mM	*mm*
Without cotyledons	0	1.0 ± 0.1
	0.1	2.2 ± 0.3
	1	3.3 ± 0.3
With cotyledons	0	3.2 ± 0.3
	1	4.1 ± 0.3

[a] Control medium, distilled H_2O.
[b] Values are $\bar{x}$ ± SE for n = 10.

authentic IAA, IAAsp, and IAGlu (Fig. 2), confirming our preliminary report (8). None of the other three unchanged peaks had relative retention times corresponding with IAA conjugates of any of the 25 amino acids tested by Hollenberg *et al.* (8).

Methyl Esterification. A further aliquot of the extract was treated with diazomethane to produce the methyl esters of carboxylic acids in the solution. The marker compounds ([^{14}C]IAA, IAAsp, and IAGlu) were present during the methyl esterification. Following HPLC, ^{3}H-labeled peaks were observed to correspond with the peaks of the presumed methyl esters of the ^{14}C-labeled markers (Fig. 3).

TLC. Segments of 200 stems were vacuum infiltered with a total of 6 μCi of [^{3}H(G)]IAA and unlabeled IAA at a concentration of 0.1 mM. The segments were incubated for 16 h in the labeling solution and extracted. An aliquot (100 μl) of the partially purified extract was subjected to HPLC. Fractions eluting between 27 and 28 min (corresponding to presumed IAAsp) and between 29 and 30 min (corresponding to presumed IAGlu) were collected. The 27.0- to 28.0-min fractions were combined and evaporated to dryness. We added 10 μl of an IAA-[^{14}C]Asp solution to the residue, followed by 0.2 ml of methanol to dissolve the radioactive metabolites. Half of the methanolic solution was applied to each of two Eastman silica gel TLC plates (without fluorescent indicator), one of which was developed with isopropanol: ammonia:H_2O (8:1:1, v/v) and the other with chloroform:ethyl acetate:formic acid (35:55:10, v/v). The 29.0- to 30.0-min fractions from the HPLC were treated similarly except that 10 μl of an IAA-

[^{14}C]Glu solution was added as a marker to the dried residue. After the solvent had migrated at least 12 cm, the chromatogram was dried. The silica gel was scraped from the plate at intervals as indicated in Figure 4, and the scrapings were eluted with Bray's solution, which was then transferred to the liquid scintillation counter. The results of the four thin-layer chromatograms are shown in Figure 4. Those parts of the chromatograms not shown in the figure were less radioactive than the least radioactive fractions included in the figure, and they have been excluded to focus attention on the active fractions. In each case, the peak of ^{3}H-radioactivity corresponded precisely with that of either IAA-[^{14}C]Asp or IAA-[^{14}C]Glu.

Time Course of Formation of Amide Bound Auxins. Several 15-segment samples were treated, each with 2.7 μCi of [2′-^{14}C]IAA (ring-labeled IAA) and unlabeled IAA at a concentration of 0.1 mM. After 0.5, 1, 2, 4, 8, 12, 16, 24, 32, and 48 h, the samples were homogenized and extracted as before. Small amounts of tritiated markers (IAA, IAAsp, and IAGlu) were added to the partially purified extracts, and HPLC and liquid scintillation counting were performed as before. The results of this experiment are displayed in Figure 5. The recovery of free IAA decreased rapidly with time. The peak cochromatographing with known IAGlu was first clearly evident after 1 h and had reached a maximum by about 4 h, while the IAAsp peak appeared at 1 h and grew until about the 8th h of incubation. The sizes of the IAAsp and IAGlu peaks remained constant until at least 48 h after the original infiltration of the tissue (Fig. 5 shows only the first 32 h, but there were no changes during the next 16 h). In the course of this experiment, we observed some changes in minor peaks of ^{14}C-radioactivity during the first 4 h of incubation, but we have not yet attempted to analyze the peaks—we have not, for example, determined whether any of the peaks seen here but not in Figure 1 are stable to base hydrolysis.

Effects of IAAsp on Elongation of the Cucumber Hypocotyl. IAAsp promoted the elongation of cucumber hypocotyl segments, whether the cotyledons were present or removed (Table I). The promotion of growth was less than half that typically observed with 0.1 mM IAA (10); and the optimal concentration of IAAsp must be >0.1 mM, which is the optimum for IAA (10).

DISCUSSION

It is evident that both IAAsp and IAGlu are formed when cucumber stems are treated with IAA. This conclusion is supported by the cochromatography of labeled IAA metabolites with known IAAsp and IAGlu in HPLC (Fig. 1) and in two TLC solvent systems (Fig. 4), by the stability of the presumed IAAsp and IAGlu peaks to mild base hydrolysis (Fig. 2), and the cochromatography of ^{3}H-labeled peaks with the methyl esters of known IAAsp and IAGlu following treatment with diazomethane (Fig. 3). Although we have presented HPLC data only for [^{3}H(G)]IAA metabolites in this report, similar cochromatography of labeled IAA metabolites with known IAAsp and IAGlu was consistently observed following initial treatment of the plants with [1-^{14}C]IAA (carboxyl-labeled), with [2-^{14}C]IAA (methylene-labeled), and with [2′-^{14}C]IAA (ring-labeled IAA). This confirms many reports of the conversion of exogenous IAA to IAAsp in plant tissues as well as two reports of the conversion of exogenous IAA to IAGlu (3, 11).

In the experiments involving incubation times of 4 h or more, we found no evidence for the formation of other conjugates of IAA with individual amino acids, although three other IAA metabolites appeared to be stable to treatment with base. These compounds might be IAA catabolites, conjugates of IAA with dipeptides, or any of a number of other metabolites. It is possible that minor amounts of another amide bound auxin appeared in the early portion of the time course experiment, but we have not explored this possibility.

The time course experiment (Fig. 5) did not shed light on the question of whether either IAGlu or IAAsp might be the precursor of the other. As noted above, it had been suggested that 2,4-dichlorophenoxyacetylglutamate and naphthaleneacetylglutamate may be the precursors of 2,4-dichlorophenoxyacetylaspartate (5) and naphthaleneacetylaspartate (6), respectively. We found that both IAAsp and IAGlu were formed rapidly and reached stable levels, with neither being obviously the precursor of the other. It is likely that IAAsp, at least, can be metabolized in the cucumber stem to yield free IAA, however, since it was a weak but significant promoter of stem growth (Table I). Feung *et al.* have compared the effects of the IAA conjugates of all 20 protein amino acids on plant growth, concluding that the growth promotions were attributable to release of free IAA from the conjugates (4). Hangarter and Good (7) have presented indirect but convincing evidence for the release of free IAA from amide bound auxins.

Experiments now in progress are directed at the effects of various treatments on the ratio of free to amide bound IAA following application of radioactive IAA. We are also testing for the presence of IAAsp and IAGlu in cucumber shoot tissue not pretreated with exogenous IAA.

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